BASIS FOR IMMUNITY TO TYPHOID IN MICE AND THE QUESTION OF "CELLULAR IMMUNITY"

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INTRODUCTION

The classical studies of Metchnikoff and his colleagues clearly demonstrated that animals of various phyla, both invertebrates and vertebrates, respond in a characteristic fashion toward the implantation or invasion of their tissues by foreign particles (36, 37). The observed response is a cellular one; phagocytic cells move toward the particle and ingest it. To Metchnikoff, this cellular response represented the basic defense mechanism possessed by most forms of life against potential parasites. After the demonstrations of "antibody" by von Behring and Kitasato (62), the field of host-defense mechanism was divided between two schools of thought; some considered the phagocytic cells functionally independent of antibody of major importance, and others regarded the formation of specific proteins complementary to the antigen as the critical feature of host resistance.

This unfortunate and unnecessary division led to a great deal of controversy which was, to some extent, resolved by the observations of Wright and co-workers (64) on the phagocytosis of bacteria by polymorphonuclear cells. They were able to demonstrate that the presence of serum (particularly serum taken from animals specifically immunized against those strains used in their experiments) greatly enhanced the phagocytosis of these bacteria by polymorphonuclear phagocytes. The substances in serum potentiating the reaction between the phagocytic cell and the bacterium were termed "opsonins." It should be borne in mind that opsonin is a purely functional term and is not descriptive of any particular class of serum proteins. Thus, any serum protein or substance which reacts with the surface of a particle in such a manner as to increase its rate of ingestion by phagocytic cells may be called an opsonin.

These initial findings have been supported by numerous studies (2, 8, 17, 23, 26, 34, 48, 63) of the phagocytosis of a variety of gram-negative and gram-positive bacteria, both by mononuclear and polymorphonuclear cells from various animal species. These studies have emphasized the interdependence of the cellular and humoral systems, the functioning of the one depending on the presence of the other. However, despite these varied observations, the concept of a cellular type of immunity distinct from and independent of antibody still dominates certain areas of the field of infectious disease. Particularly good examples of infections which are said to be controlled by an immune cellular mechanism are tuberculosis, brucellosis, and salmonella infections of mice and other animals. The causal pathogens of the above diseases are parasites of the reticuloendothelial system and are capable of multiplying within mononuclear phagocytes after ingestion. With the above infections, the main reason for postulating a type of immunity based on cells has been the inability in the past to protect animals against infection by the passive transfer of convalescent serum. Further, it has not been possible to actively immunize individuals against these infections with dead bacteria, though a high titer of specific antibody

may be produced by this means. To obtain a significant degree of protection, animals have to be vaccinated with living attenuated strains of these bacteria. This has often been taken by the proponents of cellular immunity as further supporting evidence for the existence of such a state. In vitro experiments with macrophages obtained from actively immune individuals suggest that these cells, compared with those obtained from normal nonimmune animals, are endowed with the capacity to kill or suppress the multiplication of the pathogen after ingestion, and that serum antibody plays no role in the inactivation of the parasite.

However, it would seem to us, in light of recent studies on brucellosis and of our own work, which has been concerned with the pathogenesis of mouse typhoid, that there is, as yet, no need to postulate an immune mechanism independent of antibody for these infections. Before presenting our own data, it would be pertinent to briefly review the literature concerning these infections and to critically examine the data which support the concept of an immune cellular mechanism. First, it would be well to define carefully what we think is meant by the term "cellular immunity," as used by various workers. To us, cellular antibacterial immunity implies either the production by the host of new phagocytic cells on stimulation by the parasite, or induction of existing cells by the parasite so that these cells are now capable of killing the pathogen, whereas previously they allowed it to grow intracellularly. To have any meaning at all and to distinguish it from the altered cellular state produced by nonspecific agents, such as lipopolysaccharides from gram-negative bacteria (45), cellular immunity must encompass specificity. This would imply that macrophages from an animal immune to pathogen A were capable of killing pathogen A, but their bactericidal activity toward pathogen B would not differ from that of cells from the normal nonimmune animal. Further, it should be possible to passively transfer this specific immunity with living phagocytes in the absence of free or cell-bound antibody. Killed "immune" phagocytes should be inactive in such a transfer experiment.

With this definition in mind, we might now consider each of these three infections—brucellosis, tuberculosis, and salmonellosis.

INTRACELLULAR MULTIPLICATION OF *BRUCELLA* IN MONONUCLEAR PHAGOCYTES FROM NORMAL AND IMMUNE ANIMALS

Strains of *Brucella* are intracellular parasites, and, since in the past it has been difficult to correlate immunity and antibody titer, a great deal of attention has been centered on the possibility that the acquired resistance to infection resides in the ability of the "immune" mononuclear cell to kill the ingested bacteria. In vitro studies on the interaction of various strains of *Brucella* with macrophages obtained from normal and immune animals would suggest at first sight that this indeed might be the case.

In the experiments of Pomales-Lebrón and Stinebring (44), macrophages were obtained from the peritoneal cavities of normal and immune guinea pigs by glycogen stimulation. The harvested cells were washed once and allowed to phagocytize *Brucella abortus* in the presence of 30% guinea pig serum for a period of 2 hr. The medium was then changed, and the number of bacteria associated with cells at that time was taken as the zero count. Further observations on the cells were made at the end of 20, 48, and 72 hr. Extracellular growth of bacteria was controlled by the addition of 10 μg/ml of streptomycin to the tissue culture medium. The results showed that the growth of *B. abortus* in "immune" cells was not as great over this time period as that observed in cells obtained from the peritoneal cavity of normal animals. It is interesting to observe, however, that if one calculates the apparent destruction of bacteria for the first 20 hr then both normal and "immune" cells constitute a highly efficient bactericidal system; approximately 95% of the ingested bacteria were killed by the mononuclear phagocytes from the normal animal, whereas the destruction of bacteria within the "immune" phagocytes was greater than 99%. Unfortunately, however, during this period of time there was almost a 50% loss of phagocytic cells, which makes it difficult to assess the overall reaction.

Extending these observations, Braun, Pomales-Lebrón, and Stinebring (4) found that whereas smooth strains of *Brucella* apparently proliferated within the phagocyte, rough strains showed little or no growth. Nevertheless, the number of bacteria phagocytized was greater

than that observed with the smooth strains, with the result that all the monocytes had been destroyed at the end of 48 hr. One strain of *B. abortus* (S19) was apparently killed by mononuclear cells from normal animals. "Immune" phagocytes rapidly ingested both smooth and rough strains and were eventually destroyed by the smooth strains, though the observed increase in bacteria per population of macrophages was less in the "immune" system than that observed in the normal system.

In a similar study, Holland and Pickett (21) found that *B. suis*, *B. abortus*, and *B. melitensis* multiply after ingestion by peritoneal monocytes obtained from various species of animals by glycogen stimulation. Monocytes obtained by the same method from animals previously immunized with a living vaccine of *B. abortus* or *B. melitensis* inhibited the growth of all these strains. Phagocytic cells from animals immunized with a killed vaccine were not effective. Phagocytosis of bacteria took place over a period of 5 hr in the presence of 40% heat-inactivated normal rabbit serum. Streptomycin, at a concentration of 10 μg/ml, was added after the period of time allowed for phagocytosis. The intracellular population at 5 hr after the addition of streptomycin, and therefore 10 hr after the addition of bacteria, was taken as the zero count. In this circumstance, it becomes impossible to determine the relative rates of phagocytosis in normal and immune systems; nor is it possible to calculate any killing that may have taken place during the first 10 hr of the experiment. For reasons which will be discussed later, the first hour, indeed minutes, seem to us to be the most critical period for the study of the interaction between cells and bacteria. The immune cells appeared to act in a bacteriostatic fashion in that growth of the organism did not take place nor was there any apparent killing. An interesting feature of this work (21) contained the statement that after 3 days there was obvious cell damage and a decrease in the numbers of bacteria. It was explained by the authors as being due to the penetration of streptomycin.

Recent studies by Carpenter, Fukuda, and Heiskell (7) have shown that macrophages obtained from animals immunized with either *B. abortus* or *B. melitensis* in the presence of *Brucella* organisms, lipopolysaccharide, or brucellergen are extensively damaged. More than 80% of the macrophages became detached from the walls of the tissue culture tubes within 24 to 48 hr, showing evidence of rapid cytolysis. Macrophages obtained from the spleens of normal animals showed no significant morphological changes and were similar in appearance to control cells kept in the absence of antigen.

In view of the injury caused by *Brucella* antigens to macrophages obtained from the immune animal, one cannot exclude the possibility that the apparent bacteriostatic effect of immune cells previously reported has been due to leakage of antibiotics into damaged phagocytes, especially since observations were made at infrequent intervals over comparatively long periods of time.

The observations of Elberg, Schneider, and Fong (9) showed that trypsin-treated macrophages from rabbits immunized with either BCG or living *B. melitensis* were capable of inhibiting the intracellular growth of *B. melitensis* as measured by the degree of cellular damage occurring over a period of 48 hr. However, this effect was only observed when phagocytosis of the bacteria had taken place in serum from the immune animals. If normal serum was used, then "immune" macrophages were no better at dealing with this intracellular parasite than were the phagocytes obtained from the nonimmune animal.

These experiments are very interesting, since they indicate the nonspecificity of the cellular reaction and the importance of the humoral component. Elberg (10) interpreted these results as indicating that the immune serum "exerts an important influence on the maintenance of monocyte structural integrity." This statement is ambiguous, but in the light of our own experiments we would interpret these results to mean that antibody, reacting with the bacterium, determines the rate of intracellular destruction of the organism once phagocytized (24, 26, 46). This interaction, in the case of brucellae, is reflected in the increased survival in tissue culture of infected "immune" cells as compared with infected "normal" cells.

In the past, a further argument for the existence of cellular immunity has been the

failure to immunize animals against this infection with a killed vaccine, though there may be produced by this means a high titer of agglutinating antibody against the virulent strain. The importance of these observations and their implications will be discussed later. However, Sulitzeanu (54) was able to protect animals against *B. abortus* by immunizing animals with nonliving antigens prepared from *B. suis*. In a similar study, Smith et al. (53) found that certain fetal fluids obtained from pregnant cows infected with *B. abortus* were immunogenic in guinea pigs and mice, and that good protection was afforded in these animals against subsequent challenge with the specific organism. Extending these observations, Keppie, Witt, and Smith (29) found that vaccination with cell-wall preparations or culture filtrates obtained from any of these strains of *Brucella* protects guinea pigs against challenge with any other strain of this pathogen. Any argument for the existence of cellular immunity that is based upon the inefficacy of killed vaccines must surely call for reconsideration in the light of such evidence as this.

These results obtained with nonliving antigens indicate the importance of antibody in the dynamics of these infections. There can be no doubt, however, that phagocytic cells obtained from these animals may show greater bactericidal potential, but this may be interpreted in a nonspecific manner as discussed by Rowley (45) and as the results of Elberg, Schneider, and Fong (9) also suggest.

INTERACTION OF MACROPHAGES WITH *MYCOBACTERIUM TUBERCULOSIS*

Mycobacterium tuberculosis is a notoriously difficult organism to work with and, in spite of the tremendous amount of effort that has gone into the study of the phagocytosis and intracellular fate of this bacterium under different conditions, we are still in a dilemma as to the role that humoral antibody might play in the ingestion and subsequent inactivation of this organism. Some of these difficulties have been adequately reviewed by Hanks (16). Most studies have emphasized the importance of the "immune" cell in the pathogenesis of this infection, and it will be well to review this field in the light of our present knowledge. Lurie (30) studied the fate of the tubercle bacillus in normal and previously immunized rabbits. He found that growth of the tubercle bacillus was inhibited in the immune animals. Lurie also made the interesting observation that the macrophages of the immune animal had a greatly increased phagocytic capacity for a number of particles other than the tubercle bacillus. Ingestion of particles, such as carbon, collodion, and staphylococci, was rapid compared to that observed in normal rabbits. From these in vivo studies, Lurie made the suggestion that the apparent inhibitory effect of immune cells on the intracellular growth of the tubercle bacillus could be due in part to antibody, the in vivo situation preventing one from dissociating the cellular and humoral parameters.

However, his further studies tended to deny this idea. These later ingenious experiments involved parasitizing macrophages from normal and immune rabbits in vitro, and subsequently transferring them to the anterior chamber of the eye of normal rabbits. Various combinations of cells and serum were used, and Lurie concluded that immunity to tuberculosis in the rabbit resided in some intrinsic property of the cell (31). However, in most instances the eye chambers were not examined until 14 to 20 days after the infected cells were planted, and thus we know nothing regarding the early events of cell–bacterium interaction. Further, this technique is extremely difficult to quantitate and, if one examines the data of individual experiments, it is apparent that in some instances macrophages from the normal rabbit, when phagocytizing the bacteria in the presence of normal serum, constituted as efficient a bactericidal system as that observed when "immune macrophages" ingested the organism in the presence of immune serum. Other criticisms of the technique, such as the possibility of transferring hypersensitivity and the subsequent effect of this on the tubercle bacillus, were discussed by Mackaness (32, 33).

Using an in vitro technique which allowed precise quantitation of the intracellular events, Mackaness (32, 33) found that there was no difference in the ability of macrophages from normal rabbits and those obtained from specifically immune animals to support the growth of this organism. It would have been interesting in these experiments to have studied the fate of the tubercle bacillus within normal and "immune" macrophages that had ingested the

organism in the presence of serum from the immune animal. In the experiments of Lurie (31), there is some indication that the presence of immune serum enabled normal macrophages to deal with the bacteria more efficiently. Mackaness (32, 33) also stressed the importance of the ratio of bacteria to macrophages. In many instances, the organism may be cytotoxic if ingested in large numbers, and, unless this ratio is kept small, subsequent intracellular events could be masked by death of the phagocyte and leakage of antibiotic into the internal milieu. Following the experimental technique of Mackaness (32, 33), and using trypsin-treated phagocytes, Fong, Schneider, and Elberg (11) studied the interaction between tubercle bacilli and macrophages from normal and immune animals after treatment with either normal or immune serum. These results indicated that, given a system in which immune serum was a component, there was inhibition of the multiplication of the ingested bacteria. Extending these results, they found that immune serum protected both normal and "immune" macrophages against the cytotoxic effects of certain products of this organism (12). To investigate the specificity of this reaction, rabbits were immunized with a formalin-killed suspension of *Salmonella rutgers*. Macrophages from these animals were tested for their ability to inhibit the growth of the tubercle bacillus. Phagocytosis of the bacteria took place in serum from normal animals and from animals previously immunized with BCG. It is not surprising that macrophages obtained from salmonella-immunized rabbits behaved in both these tests as did macrophages from normal rabbits. However, these experiments lend no weight to the argument that cells from animals immunized against the tubercle bacillus display specificity. It would have been interesting to have tested these latter cells against a range of bacteria unrelated to the tubercle bacillus. Where such experiments have been done, it is apparent that, although the macrophages from animals vaccinated with BCG showed a greater bactericidal potential compared with similar cells from normal control animals, this increased activity was displayed against a variety of organisms (22, 26, 55). Indeed, the early work of Elberg, Schneider, and Fong (9) suggests that there was a lack of specificity, though this was not fully explored. The results of these workers, despite the lack of precise quantitation, are interesting, for they reveal the importance of the humoral component in expressing the bactericidal potentiality of the phagocytic cell. In a similar study, Suter (56) obtained differences in the capacity of macrophages from normal and immune rabbits to inhibit the growth of this organism. He concluded, after studying the effect of immune serum and normal serum in his system, that the growth-inhibitory properties of the phagocyte were independent of the humoral component. This conclusion, however, seems unjustified, since phagocytosis of the bacteria took place in the exudate from either the normal or immune animals. In other words, immune cells always took up bacteria in the presence of immune serum. It would appear also that, in experiments determining the effect of immune serum on the bactericidal activity of macrophages from normal animals, the immune serum was added to the tissue culture system after ingestion of bacteria had taken place in the exudate from the normal animal. It is not surprising, therefore, that immune serum had no effect on the subsequent intracellular events. Elberg (10) concluded that these findings indicate that the serum factor(s), in some as yet unknown fashion, acts on the cell, protecting it from damaging effects of the tubercle bacillus. It would appear to us that these results could indicate that the serum component reacts with the bacterial cell before phagocytosis, and it is this complex which is destroyed by the cells from the immune animal. These phagocytes are in a stimulated physiological state, but their immunity (if we can use that term) is entirely nonspecific (22, 26, 55).

Studies on Salmonella Infections in Mice

Salmonella infections of animals have interested students of infectious disease for a number of years. The mouse is probably the most widely used laboratory animal, and we are fortunate that several strains of salmonella are natural pathogens of this species. Experimental infections with these bacteria have served as models to investigate various epidemiological problems and to study the basis for natural resistance and susceptibility to infection between species and between strains within the same species of animals (14, 15, 40). Early studies of the pathogenesis of *S. typhimurium* infections in mice showed that the injected bacteria are cleared

from the circulation and may be found mainly in the liver and spleen. Investigations of the fate of the bacteria in these organs showed that, whereas the population of a virulent strain of *S. typhimurium* increases, that of an avirulent strain declines; the rate of killing is related to the virulence of the organism (41, 43).

To study the kinetics of this infection, most workers have resorted to studying in vitro the phagocytosis of *S. typhimurium* by macrophages from the mouse peritoneal cavity and their subsequent intracellular fate. The mouse is a particularly suitable animal for this purpose, since one may obtain pure cultures of macrophages without the use of various irritants, such as glycogen or oil. There is a certain disadvantage in working with phagocytic cells that have been obtained by such means, since the metabolism of these cells is altered and their phagocytic and bactericidal potential is abnormal (45).

The results of in vitro studies from various laboratories have been contradictory, and it is necessary to examine some of these data critically to arrive at some logical conclusion. Furness (13) found that both virulent and avirulent strains of *S. typhimurium* were phagocytized equally well by mouse peritoneal macrophages. After ingestion, both strains were killed at the same rate for the first 3 hr, but the virulent organism subsequently multiplied while the avirulent one was eliminated. These results are in striking contrast to those obtained by other investigators. Jenkin and Benacerraf (26) found that, in the presence of normal mouse serum, peritoneal macrophages phagocytized avirulent strains of *S. typhimurium* much more readily than virulent strains. The subsequent intracellular survival of virulent organisms under these circumstances was very high. In contrast, though greater numbers of avirulent bacteria were taken up by these cells, only very small numbers persisted at the termination of the experiment. If, however, the virulent bacteria were first treated with a specific antiserum, then the phagocytosis and intracellular survival of these bacteria was similar to that observed with the avirulent strain after treatment with serum from the normal mouse. These observations with a specific antiserum support the earlier findings of Whitby and Rowley (63) and stress the importance of antibody in the cell–bacterium interaction. More recently, it has been found that antibody not only determines the rate of uptake of those bacteria but also the subsequent intracellular events (24), the rate of killing being limited by the presence or absence of antibody. These in vitro findings, although carried out under obviously artificial conditions, are supported by in vivo observations. Avirulent strains of *S. typhimurium* are eliminated much more rapidly from the circulation of the mouse than are virulent ones (Auzins, *personal communication*; 27). Furthermore, if virulent bacteria are previously well opsonized, the mouse is able to deal with them initially as efficiently as it deals with avirulent strains (28). So far, then, both in vivo and in vitro experiments support the general idea that, while antibody in itself is not lethal, in combination with phagocytic cells it constitutes a highly efficient bactericidal system.

In further analyzing the pathogenesis of mouse typhoid, we are faced with a dilemma which appears at first to contradict the importance of the foregoing observations. Firstly, unless one carefully chooses the challenge dose of bacteria, it has been found that preopsonized bacteria are as lethal for the mouse as are nonopsonized bacteria. In most instances, the best that one can measure in terms of protection is an increased survival time, rather than a difference in overall mortality (*unpublished data*). Secondly, it has been found that mice dying of this infection, and showing a persistent bacteremia, have a high titer of opsonins against the challenge organism and are capable of eliminating from the circulation a second challenge dose of isotopically labeled bacteria much more rapidly than do uninfected control animals (Table 1). Careful studies on the bacteria circulating prior to death would suggest that

TABLE 1. *Clearance of the virulent strain of Salmonella typhimurium labeled with the isotope P^{32} from the circulation of mice dying from infection with the same virulent strain**

Time after challenge with C5	Phagocytic index K (unopsonized bacteria)
days	
5	0.036
6	0.08
7	0.05
10	0.19
11	0.28
Controls	0.020

* Rate of clearance expressed as the phagocytic index K for a dose of bacteria of 10^9 per 100 g.

these bacteria are similar in their antigenic structure to those used in the initial challenge, so that their persistence is not obviously related to any change in surface antigens as has been reported for *Pasteurella pestis* (5, 6). Thirdly, despite numerous attempts at specific immunization, it has been found virtually impossible in the past to protect mice against this infection by use of killed vaccines, although such vaccines provoke a relatively high titer of anti-O antibody. Living attenuated strains, on the other hand, give very good protection; it has also been found that survivors of an infection are highly resistant to subsequent reinfection (18, 19, 20). These survivors, while appearing outwardly normal, may harbor the organism in their reticuloendothelial system for varying periods of time (19, 20).

Before considering this latter aspect of the infection, i.e., the carrier state, it would be best to offer some explanation for parts one and two of the dilemma facing us. In vitro experiments concerned with the intracellular survival of these bacteria give us a very good clue as to the situation in vivo. It is apparent from these, together with the results of numerous other workers studying the phagocytosis and intracellular survival of a variety of bacteria, that a population of macrophages or polymorphonuclear cells is not homogeneous in respect to their ability to kill bacteria (8, 17, 26, 34, 42, 48, 63). It is also obvious from these studies that the bactericidal potential of the population of cells is evident only for the first hour or minutes (depending on the opsonic conditions) after infection of the tissue culture system. Hence, if one ignores this initial stage of the reaction, it is likely that the further events observed are occurring in incompetent cells. These results, although a true reflection of the intracellular events within these cells at that time, may lead to erroneous conclusions regarding the competence of the population as a whole.

There is good evidence from in vitro studies that phagocytosis, even under the best opsonic conditions, does not necessarily lead to death of the ingested bacteria. If we can assume that this heterogeneity also exists in vivo, then it would mean that a percentage of the ingested opsonized bacteria would survive and multiply within host phagocytes. Any daughter cells resulting from this multiplication would, of course, be unopsonized, and hence would be similar to a nonopsonized challenge dose. However, since antibody determines the overall rate of killing in this system, one might expect that a greater percentage of the challenge dose with opsonized bacteria would be killed than if the challenge consisted of entirely unopsonized bacteria. Thus, the overall effect of challenging with preopsonized bacteria would be the same as if one gave a much smaller dose of unopsonized bacteria. The antibody response to this organism, as measured by an increase in the opsonic titer of the serum, is not apparent until 3 to 5 days after challenge. Under these circumstances, the host would be faced with a population of bacteria large enough to kill it from endotoxemia. This situation was critically examined by Rowley (45) and need not be further emphasized. There is also the possibility, which has been discussed elsewhere, that in these susceptible animals there could also be a delay in the production of certain protective antibodies (25, 47).

The heterogeneity in bactericidal activity of the population of phagocytic cells would explain the inability to protect animals against this and similar infections by the passive transfer of immune serum. In many cases, the serum has been given once prior to challenge and then no more. If one takes into consideration the half-life of the immune globulin in the face of the host's antibody response to the injection of foreign protein, it is likely that it would not persist long enough to measurably affect the outcome of the infection. This inability to transfer immunity by serum from immune animals, together with the fact that killed bacteria appear to be ineffective in inducing immunity (despite an antibody response to the killed vaccine), has led to the supposition that the immune state is expressed by some specific property of the phagocytic cell in a fashion entirely analogous to putative examples of cellular immunity. For salmonella infections, this idea stems largely from the results of Japanese workers who have studied extensively the phagocytosis and intracellular survival of *S. enteritidis* within peritoneal macrophages from normal and immune animals (49, 59, 60, 61). They also claim that immunity may be passively transferred by phagocytic cells, but not by serum, from immune animals (38, 50, 51, 52, 57). Their in vitro studies take into account only those intracellular events occurring 24 hr and later after infection; hence, early events have been ignored. It is worth noting in this respect that the authors mention that "intracellular bacteria seemed almost to disappear on the first day of incubation," though the

subsequent events were considered more important. In general, the lack of good quantitative data and inadequate details of methods make these papers difficult to evaluate. However, in the light of their work, the possibility of an immune state dependent on cells needs careful consideration. We have attempted to evaluate their results in relation to our own study of the basis for immunity to *S. typhimurium* and of the ability to transfer passively this state by cells or serum from immune animals (*unpublished data*).

Immunological Basis for Resistance to Salmonella typhimurium

It has been known for some time that animals that have recovered from infection with *S. typhimurium* or have been vaccinated with a living nonvirulent strain of this organism are highly resistant to further reinfection. The basis for this immunity is not known, though it has been inferred by Hobson that specific antibody is unlikely to play a role in this protection in view of the fact that killed vaccines, though producing O-agglutinating antibody, are ineffective in protecting animals against this disease (18, 19, 20).

To investigate this problem more fully, a large group of mice was infected intraperitoneally with 10^6 bacteria of the avirulent strain. At various time intervals after this challenge, groups were reinfected with 1000 lethal doses of the virulent strain, and their fate was followed for a period of 28 days. With each challenge group, a batch of mice was examined for their ability to clear a dose of P^{32}-labeled virulent bacteria from the circulation. Liver and spleen weights were also recorded for this latter group of animals. The results of this experiment are given in Table 2 and Fig. 1. It is apparent that the appearance of resistance to this infection closely parallels the increase in opsonic titer of the serum. It is interesting to record that there was no significant increase in the weight of the spleen and liver of these animals during the period of this experiment. The protective effect of immunizing with the attenuated strain was dose-dependent, the delay in the appearance of immunity at the lower dose levels being related to the opsonic titer of the serum of these animals at the time of challenge (Table 3). Macrophages harvested from the

TABLE 2. *Resistance of mice immunized with an attenuated strain of Salmonella typhimurium to further challenge with the virulent strain*

"Carrier" period	Challenge dose	Mortality at 28 days	
		Test animal	Control
		%	%
24 hr	5×10^2	88	90
48 hr	2.1×10^4	75	100
96 hr	1.6×10^4	75	100
1 week	4×10^3	10	100
2 weeks	1.6×10^4	40	100
1 month	1.9×10^4	50	100
2 months	1.9×10^4	32	100
4 months	1.4×10^4	45	100

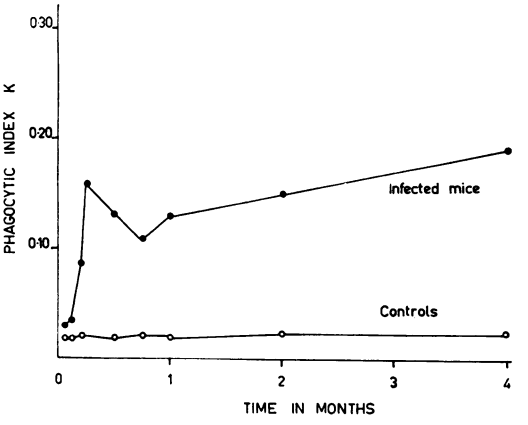

FIG. 1. *Increase in opsonic titer of the serum of mice previously infected with an attenuated strain of Salmonella typhimurium.*

TABLE 3. *Effect of various doses of the attenuated strain on the appearance of immunity to further challenge with the virulent strain of Salmonella typhimurium*

"Carrier" period	Dose of avirulent strain	Challenge dose	Mortality (28 days)
			%
1 week	100	1.4×10^4	85
	1000		72
	10,000		58
	Controls		100
2 weeks	100	3×10^3	100
	1000		63
	10,000		50
	Controls		100
1 month	100	2×10^3	72
	1000		20
	10,000		40
	Controls		100

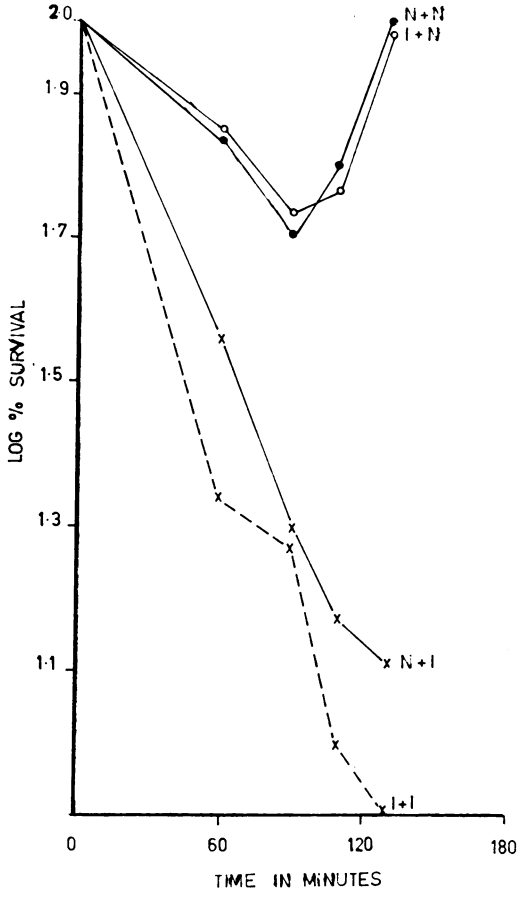

FIG. 2. *Intracellular survival of a virulent strain of Salmonella typhimurium within peritoneal macrophages from normal and specifically immune mice after opsonization with serum from either normal or immune animals. Symbols:* ●——● *(N + N) = normal macrophages + normal serum;* ○——○ *(I + N) = immune macrophages + normal serum;* ×——× *(N + I) = normal macrophages + immune serum;* ×——× *(I + I) = immune macrophages + immune serum.*

peritoneal cavities of immune mice 4 months after the immunizing dose were similar in their phagocytic and bactericidal activities toward the virulent strain to macrophages from normal animals. However, macrophages from both groups of animals phagocytized and killed the virulent strain extremely efficiently if these were first treated with serum from the immunized group (Fig. 2). The opsonic activity of the serum of these animals was specific in that strains of *Escherichia coli*, staphylococcus, and *Klebsiella*

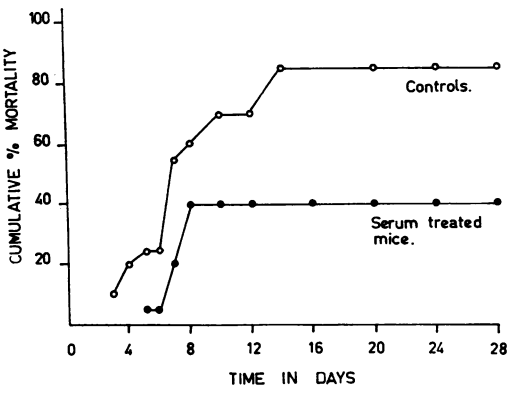

FIG. 3. *Effect of passive transfer of immune serum on the susceptibility of mice to Salmonella typhimurium infection.*

pneumoniae were cleared from the circulation of these mice with the same order of efficiency as that observed in normal mice.

Passive Transfer of Immunity with Serum from Immune Animals

A batch of pooled serum was obtained from immunized mice at a time when previous experiments had shown that one would expect these animals to be highly resistant to challenge with the virulent organism. Normal mice were then injected intravenously with 0.2 ml of this serum every day for a period of 3 days prior to an intraperitoneal challenge with 1000 lethal doses of the virulent strain which had been preopsonized with the same serum. After this challenge, the mice were given immune serum daily for an additional 5 days. The results of this experiment showed that under these conditions it was quite possible to transfer immunity passively with serum, protection being measured both as increased survival time and overall mortality (Fig. 3).

Passive Transfer of Immunity by Cells from the Peritoneal Cavity of the Immune Animal

While the Japanese workers have not successfully induced immunity by serum transfer, they have obtained some degree of protection by the passive transfer of "immune" cells (49, 59, 60, 61). Peritoneal exudates were induced in mice previously immunized with a live vaccine. The cells were washed twice and then transferred to the peritoneal cavities of normal mice. These mice were challenged with the virulent strain and

their fate was followed for a period of 28 days. Protection was observed only in animals infected 4 days after the transfer of cells. They also found that whenever successful transfer induced immunity some living bacteria were present in the cell suspensions. These results suggested to us that the observed immunity could be explained by the passive transfer of antibody-producing cells.

The observed latent period before any immunity was apparent would suggest the production of antibody by the transferred cells or an active immunization due to the presence of antigen in the cell population. Although the authors claim that the observed immunity was due to the transfer of monocytes, it is known that exudates from the peritoneal cavity may contain a high percentage of lymphocytes (Mackaness, Symp. Soc. Gen. Microbiol., *in press*), and the possibility of antibody production by these cells was not considered. Ultrasonic treatment for a short period of time (2.5 min) did not destroy the ability to transfer immunity, although no intact cells survived this procedure. Further exposure of the cells to ultrasonic vibrations (5 min) completely destroyed the activity of the exudate cells (59). It would appear from these experiments that the transferred immunity is independent of living cells and therefore can no longer be considered, according to our earlier definition, as cellular immunity. The possibility remains that these authors were injecting antibody bound to the disrupted cells and that this cell-associated antibody was the significant factor in immunity, for recent experiments would suggest that antibodies may be firmly bound to cell surfaces (3). It is this association of antibody and cell which has been exploited in the studies of antibody production by Nossal and Mäkelä (39).

Experiments were designed to explore the various possibilities set out above. A different technique was used to evaluate the state of immunity after transfer of the cells. In principle, normal mice were injected intraperitoneally with cells or serum (or both) from mice that had been infected 2 weeks previously with a living attenuated strain of *S. typhimurium*. After this, the mice were challenged intraperitoneally with a virulent, streptomycin-resistant mutant of the virulent parent. At various time intervals after challenge, mice were killed, and counts were made on the peritoneal washings. A suitable sample of the washout was plated onto nutrient agar containing streptomycin, thus enabling one to distinguish between the challenge dose and bacteria that might have been carried over with the cells from the immunized mice.

In all of the experiments, 5×10^6 to 10^7 living cells or their equivalent were transferred to each mouse.

Cells from immune mice were injected intraperitoneally into one group of normal animals. A second group received a 1:5 dilution of serum from these same animals. After 5 min, the mice were challenged with the virulent organism, and its fate was followed. In both groups, more than 90% of the challenged bacteria were killed in 60 min. In control mice that had received either cells or serum from nonimmune mice, there was no killing of the virulent bacteria over this time period.

Cells from the immune mice were treated with 0.5 mg/ml of trypsin for 45 min and washed twice with tissue culture medium. These trypsin-treated cells were injected into one group of mice, while a second group of mice received trypsin-treated cells that had been suspended in a 1:10 dilution of immune serum for 30 min. The latter cells were centrifuged gently and washed twice before transfer. Mice were then challenged with virulent bacteria as in the previous experiment. It was found that only 50% killing had taken place in the peritoneal cavities of mice receiving trypsin-treated cells, whereas more than 98% of the bacteria had been killed in those mice receiving trypsin-treated cells that had been in contact with immune serum prior to transfer. Prolonged trypsin treatment destroyed completely any immune activity possessed by these cells.

The above experiments indicate that immunity may be transferred by cells, as measured by the increased capacity of the normal animal to deal with the virulent bacteria. This activity is destroyed by trypsin, but trypsin-treated cells may regain their "immunity" if suspended for a short period in immune serum. There is a strong possibility that this trypsin-labile factor is cell-bound antibody. Recent experiments have shown that some of the antibodies against *S. typhimurium* in the serum of immune mice are macroglobulins which have a high affinity for surfaces. It has also been found that the ability to transfer immunity by cells is closely associated with the period of 19S antibody production. Cells obtained from mice at a stage when 7S antibody is mainly present are ineffective (*unpublished data*).

Cells from the peritoneal cavity of immunized mice were frozen and thawed several times. It was found that injection of the frozen-and-thawed mixture into the peritoneal cavities of normal mice greatly increased the rate of killing of injected virulent bacteria. A sample of the frozen-and-thawed cells was centrifuged, and the cell-free supernatant tested for its "immune" activity. The results of the experiment showed that some activity, which was trypsin-labile, was present in the supernatant.

Treatment of the frozen-and-thawed deposit with 2 M urea for 5 min gave an extract which was capable of opsonizing the virulent strain, as indicated by the increased rates of phagocytosis and killing. These experiments were repeated with cells from nonimmune mice with completely negative results. Similar experiments with cells obtained from mice immunized with *S. enteritidis* gave almost identical results. These experiments strongly support the contention that the ability to transfer immunity with cells is associated with their content of cell-bound antibody, particularly in those instances where immunity has been conveyed by means of nonviable cells.

Thus far, the experimental results support the idea that immunity to mouse typhoid is dependent on the production of specific antibody, and it is this dependence which determines the rate of ingestion of the virulent bacteria by the phagocytic cells and their subsequent killing.

Immunization of Mice with Killed Vaccines

In the past, it has not been possible to protect mice against this infection with a killed vaccine. Some of this failure could be accounted for by inadequate antigen dosage, though in other instances a high titer of antibody to the O somatic antigen was obtained. In view of our previous findings and conclusions, one would expect that a suitably prepared nonliving vaccine given in adequate amounts would protect mice against *S. typhimurium*. Recently, Auzins (*personal communication*) found that the virulent strain of *S. typhimurium* used in our studies possesses a heat-labile antigen. If vaccines are prepared with bacteria that have been killed by methods which preserve this heat-labile antigen, it is possible to protect mice against this infection (*unpublished data*).

The Question of Cellular Immunity

The infections that have been reviewed in this paper have at least one factor in common: the causal organisms are intracellular parasites of the reticuloendothelial system. However, it is an observed fact that the host can be modified in such a manner that the intracellular environment of the phagocyte no longer favors the survival of the ingested bacterium. It is the reason for this altered response on the part of the cell toward the parasite that has concerned us in this paper.

By analogy with the results obtained from an in vivo and in vitro study of the pathogenesis of *S. typhimurium* infection in mice, we have suggested that immunity to both tuberculosis and brucellosis resides in the capacity of the host to produce antibody against these parasites. It is the production of antibody and its reaction with the bacterium which determines its rate of phagocytosis and destruction within phagocytic cells. However, there is good evidence that the phagocytic cells themselves may be altered by these infections, producing in them a higher rate of metabolism and an increased enzyme content (1, 58).

This increase in metabolism is reflected in the greater phagocytic and bactericidal potential of these cells, which is expressed only in the presence of opsonized bacteria. Furthermore, the increased phagocytic and bactericidal activity of such stimulated phagocytes is nonspecific. This is evident from a number of studies which have already been discussed.

More recently, Mackaness (35) investigated the pathogenesis of *Listeria monocytogenes* infection in mice. During the first 3 days of the infection, the bacterial population of the liver and spleen increased. On the fourth day, in the infected mice there was a change in the pattern of multiplication of the bacteria, the population of which in the liver and spleen rapidly declined.

From evidence based on histology and an elegant in vitro assay of the reaction between macrophages and *L. monocytogenes*, it would appear that the phagocytic cells of the host are modified so that the parasite is rapidly killed after ingestion. However, the bactericidal activity of these phagocytes is nonspecific. They will, for a time, destroy *B. abortus* much more effectively than will similar cells from normal mice. The possibility that cell-bound antibody may play a role in the reaction has not been ruled out (Mackaness, *in press*).

The results with the normal macrophages are very similar to those obtained when macrophages have been allowed to phagocytize other strains of

bacteria in the absence of serum. The numbers of bacteria ingested are very few, and of those ingested a very high percentage survives and multiplies within the cell (24, 46). In view of the evidence that some types of antibody may be firmly bound to cells, it would be extremely interesting to know whether the difference observed in vitro between the ability of macrophages from normal and immune animals to kill *Listeria* could be accounted for by the increased metabolic activity of the "immune" macrophages together with the presence of cell-bound antibody.

The mechanism by which antibody affects the intracellular survival of bacteria is at present unknown, but some of the possibilities have been discussed by Rowley (45).

Experiments concerned with the immunization of mice against *S. typhimurium* indicate the importance of antibody to a certain heat-labile antigen(s). At present, the chemical nature of this antigen is not known, though preliminary experiments would suggest that virulence in this species may depend on the quantity of this antigen. Investigations by Auzins (*personal communication*) indicate that, if this antigen is destroyed, then normal mouse serum is highly opsonic for the bacteria, whereas under normal conditions mouse serum is poorly opsonic (26). These results seem to indicate that this antigen may predominate over other antigenic sites on the surface of the bacterial cell either by steric hindrance or by actually covering part of the surface.

In conclusion, we feel that in our present state of knowledge there is no good experimental evidence which excludes the role of antibody in immunity to these bacterial infections. Whilst it is obvious that one cannot ignore the importance of the phagocytic cell in determining the fate of bacteria, the expression of their bactericidal activity and their ability to identify the particle as foreign is dependent on the presence of antibody.

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